EVALUATION OF THE CURRENT STATUS OF THE CELLULASE PRODUCTION TECHNOLOGY

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SUMMARY

The present report completes on time the milestone entitled "Complete assessment of cellulase production status". It presents an evaluation of the current status of research and development in cellulase enzyme production, based on a thorough review of the pertinent international literature. Taking into consideration all this information, the report exemplifies the critical issues in cellulase production and identifies strategies that will lead to a technically and economically feasible process for cellulase synthesis and utilization. The ultimate goal is to recommend research directions that need to be pursued at SERI in order to establish a cellulase production technology, which is compatible with the other units of the biomass conversion process and provides low-cost cellulase.

The cost of cellulase is a significant factor in the economics of the biomass-to-ethanol conversion technology. As a result, it has a direct impact on the cost of the ethanol produced and needs to be minimized. Cellulase can be either obtained from out-of-house suppliers or synthesized in house. The ethanol technology we are developing, however, cannot afford the risk of fluidity in quality, availability, and cost of the enzyme provided by outside manufacturers. It is therefore recommended that research be carried out at SERI to develop an enzyme production process.

The key to producing inexpensive and highly active cellulase lies in a combination of critical factors: Improved enzyme quality, enhanced enzyme productivity and yield, prolonged enzyme lifetime, and minimal cost of media and substrate. The literature reports that several organisms, both mesophilic (20-45°C) and thermophilic (>45°C), have been employed for cellulase synthesis to date, using as substrates soluble (lactose, cellobiose, xylose) and insoluble (cellulose, hemicellulose) carbohydrates. Defined or undefined (rich) media composed of nitrogen, phosphorus, magnesium, and calcium sources, trace elements, and vitamins have been developed to optimize enzyme productivity. For the same purpose, the temperature and pH of the fermentation have been manipulated in patterns depending on the microorganism used.

Batch, fed-batch, and continuous modes of operation have been evaluated. A fed-batch process using the mesophilic fungus *Trichoderma reesei* RUT-C30 and hardwood pulp ball-milled cellulose has led to the highest performance reported to date, an enzyme concentration of 57,000 IU/L and a production rate of 427 IU/L/h. Another *T. reesei* strain has been successfully cultivated in a batch 3,000 liter fermentor in a pilot plant scale enzyme production attempt.

From the literature overview it is evident that the first step towards developing our own cellulase production process is solid understanding of the kinetics of cell growth and enzyme synthesis. The most promising currently available microorganism will be employed. *T. reesei* strains are recommended for the initial research phase, since they are the best cellulase producers. However, bacterial candidates should also be examined after an initial selection screening, because they can reach high cell concentrations and thus high productivities and yields. Moreover, it is recommended that anaerobic fungi be also studied, because of the high specific activity of their cellulases; their use will eliminate the cost of aeration required for enzyme production. Because of time limitations, decisions about novel ideas and alternative proposals should be made rationally and swiftly with a certain amount of experimental work.

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INTRODUCTION

The dependence of the United States economy on imported oil renders the country vulnerable to fluctuations in the oil supply and price. The continued instability in the Middle East underlines the need for development of alternative energy sources in order to uncouple the growth of our economy from unpredictable foreign markets. The utilization of cellulose for synthesis of alternative fuels, such as ethanol, to replace or supplement gasoline seems to be the most promising technology that can provide a permanent solution to our energy needs. Hence, lignocellulosic materials have recently received increased attention as a source of cellulose, the most abundant renewable resource on earth with an annual production of approximately 4×10^{10} tons (Coughlan, 1985). Besides its abundance, cellulose is readily available from agricultural residues, forestry products and wastes, pulp and paper industry wastes, and municipal wastes. In addition to ethanol, other commodity and specialty chemicals can also be produced from cellulose including xylose, acetone, acetate, glycine, organic acids, glycerol, ethylene glycol, furfural, and animal feeds (Ryu and Mandels, 1980).

Lignocellulosic biomass consists primarily of cellulose, hemicellulose, and lignin, at a typical weight ratio of 50:25:25. Although rich in carbohydrates (cellulose and hemicellulose), lignocellulose is an insoluble substrate with a complex structure, which makes its conversion into fermentable sugars and subsequently into ethanol difficult. In a simplified representation, lignocellulose can be depicted as a network of cellulose embedded in a sheath of hemicellulose and lignin. Bondings among cellulose chains present additional constraints to the hydrolysis of cellulose to sugars. Nevertheless, using cellulose-hydrolyzing enzymes (cellulases) and ethanol-producing microorganisms, cellulose and hemicellulose can be converted first to fermentable sugars (glucose and xylose, respectively) and eventually to ethanol. Extensive research in the past decade has shown that the simultaneous saccharification and fermentation (SSF) of cellulose in a single step is the most effective and economical way to convert cellulose into ethanol.

The current status of lignocellulose bioconversion technology is based on five major steps (Figure 1): (a) Pretreatment of lignocellulose, using chemical, physical, or mechanical means to disrupt its structure and render it more accessible to enzymatic attack, (b) Enzyme production using organisms that secrete cellulase, such as fungi or bacteria, (c) Fermentation to convert xylose, derived from hemicellulose during pretreatment, into ethanol, (d) SSF to convert cellulose to cellobiose and then to glucose by cellulase and fermentation of glucose to ethanol by yeasts or bacteria, and (e) Ethanol recovery from the SSF effluent.

It becomes evident from this analysis that cellulase production plays a major role in the overall process. According to the current SERI biomass conversion process design, the cellulase production unit contributes 6% of the total capital cost of an ethanol plant (Figure 2) and 4.5% of the final cost of ethanol (Figure 3). Moreover, cellulase production not only comprises a step of the process by itself, thus influencing the economics of the proposed technology, but also affects the kinetics and economics of the SSF step, the most costly unit of the bioconversion process (Figures 2 and 3). Since commercialization of the bioconversion technology depends on its economical feasibility, it is imperative to minimize the cost of cellulase. There are, in general, two ways to achieve that: (a) By increasing the enzyme productivity of the employed microorganism and (b) By decreasing the enzyme needs of the SSF process. Increase of enzyme productivity requires identification of microorganisms that grow fast and synthesize and secrete significant amounts of cellulase or cellulase of high specific activity and stability; alternatively, the fermentation conditions need to be optimized in order to maximize enzyme productivity. Decrease of enzyme needs requires involves understanding of the factors that affect the catalytic properties of cellulase and its inactivation and improvement of the SSF conditions with regard to both enzyme activity (faster hydrolysis) and stability (long-term utilization).

The scope of this milestone report is to assess the current status of research and development in cellulase enzyme production and present critical issues of cellulase production that have a major impact on the biomass to ethanol bioconversion technology in order to identify strategies that will lead us towards optimization of cellulase synthesis and utilization.

THE SUBSTRATE: CELLULOSIC BIOMASS

Cellulose is an insoluble, high molecular weight, linear polymer of D-glucose residues linked by β-1,4-glucosidic bonds. It is the most abundant natural polymer with a degree of polymerization usually varying between 3,500 and 10,000 (Reese et. al., 1972). Cellulose chains, held together by hydrogen bonds among hydroxyl groups of adjacent molecules and van der Waals forces, form insoluble elementary fibrils (protofibrils) approximately 4 nm wide, 3 nm thick, and 10 nm long (Fan et. al, 1980). Several protofibrils, in turn, are associated together by hemicellulose (a xylose polymer) to form microfibrils, approximately 25 nm wide (Fan et. al., 1980). Lamellae of microfibrils are surrounded by layers of lignin (a phenylpropane polymer) and hemicellulose that protect cellulose from enzymatic attack. Microfibril regions of dense interchain bonding form crystalline areas, whereas regions with less ordered structure present a smaller degree of crystallinity (amorphous cellulose). It is this crystalline structure that renders cellulose insoluble and resistant to enzymes and chemical reagents. In contrast, the looser structure of amorphous cellulose makes it more readily hydrolyzable.

As a means of increasing the digestibility of cellulose, cellulosic biomass is pretreated mechanically, physically, and/or chemically prior to being incubated with cellulase. Mechanical pretreatment methods include the use of hammer mills, ball mills, and roll mills to increase the surface area and reactivity of the substrate; although effective at rendering cellulose digestible, these methods are energy intensive. The major physical pretreatment techniques are steam, ammonia, and carbon dioxide explosion, which result in significant reduction of the biomass particle size and therefore in an increase of the substrate surface area; alternatively, supercritical fluids have been used for size reduction. Chemical pretreatments include exposure of cellulose to alkali (NaOH) and dilute acid hydrolysis. The use of dilute acid (0.82% w/w H_2SO_4) has proved to be efficient at improving significantly the accessibility of cellulose to cellulase (Grohmann et al., 1985).

Some pretreatment techniques, such as the dilute acid hydrolysis, also result in solubilization of the hemicellulose fraction of biomass. This has two beneficial effects: (a) It increases the carbohydrate output of biomass in the form of xylose, and (b) It frees cellulose from its protective hemicellulose matrix, thus making it more accessible to cellulase. Unfortunately, most of the pretreatment methods leave lignin largely intact. The presence of lignin is a major concern to the biomass conversion process, since it both prevents cellulase from reaching cellulose and also adsorbs cellulase components (endoglucanase and exoglucanase), making them unavailable for cellulose hydrolysis. In addition, lignin contributes significantly to the solids concentration in process streams, rendering their pumping problematic and expensive. The use of harsh conditions to dissolve lignin, such as incubation of biomass with NaOH at high temperatures, does not solve the problem, because it is expensive and results in significant cellulose decomposition.

THE ENZYME: CELLULASE

Sources, Composition, and Properties

Cellulases, the enzymes that have the ability to degrade cellulose, perform a crucial task in the SSF process by catalyzing the hydrolysis of cellulose to soluble carbohydrates. They are primarily synthesized by fungi and bacteria, both aerobically and anaerobically. The aerobic mesophilic fungus *Trichoderma reesei* and mutants of it have been the most intensely studied sources of cellulases. Other fungal cellulase producers include *T. viride, T. lignorum, T. koningii, Penicillium spp., Fusarium spp., Aspergillus spp., Chrysosporium pannorum,* and *Sclerotium rolfsii* (Enari and Markannen, 1977; Augustin et al., 1981; Margaritis and Merchant, 1986). Fungal cellulases are glycoproteins that require no cofactor for enzymatic activity. They can be kept refrigerated or frozen in freeze-dried form, precipitated with ethanol or acetone, or in fermentation broths for years with no significant loss of activity (Ryu and Mandels, 1980). In contrast, incubation of the enzyme at 50°C and pH 4.8 (optimal cellulase activity conditions) in the absence of substrate for 28 days results in 50% loss of enzyme activity (Ryu and Mandels, 1980). On the other hand, cellulases from thermophilic fungi, such as *Thermoascus aurantiacus*, remain stable and active at temperatures higher than 50°C (Feldman et al., 1988).

In reality, cellulase is not a single enzyme but a multicomponent enzyme system with variable composition depending on its source. In general, cellulases secreted by fungi are believed to consist of three major classes of components: (a) 1,4-β-D-glucan glucanohydrolases (**endoglucanases**; EC 3.2.1.4), (b) 1,4-β-D-glucan cellobiohydrolases and 1,4-β-D-glucan glucohydrolases (**exoglucanases**; EC 3.2.1.91 and 3.2.1.74, respectively), and (c) β-D-glucoside glucohydrolases (**β-glucosidases**; EC 3.2.1.21). The reported molecular weights of cellulase components range from 5,600 to 89,000 (Feldman et al., 1988). Endoglucanases and exoglucanases adsorb to the surface of cellulose particles in order to initiate hydrolysis, whereas β-glucosidases are soluble enzymes. Even within the same organism, multiple forms of the cellulase components with differing substrate specificity have been identified. For example, the *T. reesei* cellulase consists of two endoglucanases (EG I and II) and two exoglucanases (CBH I and II); CBH I is the most abundant component of that enzyme. Among the numerous fungal cellulases, those synthesized by *Trichoderma* have the advantages of possessing all three cellulase components, being more resistant to chemical inhibitors, and exhibiting stability at 50°C. On the other hand, however, they have low specific activities and low β-glucosidase levels, they are sensitive to product inhibition, and are slowly inactivated at 50°C. At the optimal conditions of cellulase activity (50°C and pH 4.8), the endoglucanases are much more stable and resistant to chemicals than the exoglucanases (Reese and Mandels, 1980).

Several bacteria have also been identified that produce extracellular cellulases, such as *Acidothermus cellulolyticus*, *Micromonospora bispora*, *Bacillus* sp., *Cytophaga* sp., *Streptomyces flavogriseus*, *Thermomonospora fusca* and *T. curvata*, *Cellulomonas uda*, *Clostridium stercorarium*, *Acetivibrio cellulolyticus*, and *Ruminococcus albus*. In some bacteria the produced cellulase is cell-bound (e.g. *Cytophaga* sp.), while in others it is extracellular (e.g. *Cellvibrio vulgaris*), or in both forms (e.g. *Cellvibrio fulvus*). In some cases the localization of cellulase was reportedly affected by growth conditions and the age of the culture (Yamane et al., 1971; Berg, 1975). Bacterial cellulases have a different composition from their fungal counterparts; cellulolytic bacteria produce endoglucanases and either cellobiose phosphorylase (which catalyzes the conversion of cellobiose to glucose and glucose-1-phosphate) or β-glucosidase or both (Park and Ryu, 1983).

Enzyme synthesis

Cellulase is an inducible enzyme complex with cellulose, cellobiose, lactose, sophorose, and other readily metabolizable carbohydrates acting as inducers for virtually all cellulase-synthesizing organisms studied to date. However, the actual inducing molecule remains unknown, possibly a β-glycoside (Mandels and Reese, 1957). Sophorose is required at very low concentrations, whereas other inducers are needed at high levels (Sternberg and Mandels, 1979). Furthermore, the required induction time for sophorose is only about two hours, in contrast to other inducers that need to be present in the medium for about 30 hours before cellulase synthesis (Ryu and Mandels, 1980).

Cellulase expression is subject to catabolite repression by glucose at concentrations higher than approximately 0.1 g/L (Ryu and Mandels, 1980), believed to occur at the translation level (Nisizawa et al., 1972). When *T. reesei* was cultivated in 5 g/L glucose, cellulase was synthesized only after glucose was almost completely consumed (Mandels and Reese, 1957). In that study, lactose and cellulose were found to be an excellent cellulase inducer, cellobiose was a poor inducer, whereas glucose, starch, and maltose did not induce enzyme synthesis. In addition, the presence of metallic ions (Ca^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+}) was required for cellulase production, but not for cell growth. Catabolite repression has been also observed with other soluble sugars, such as glycerol (Montenecourt and Eveleigh, 1977; Hoffman and Wood, 1985). Moreover, these researchers reported differential effects of the repressor on the expression of the various cellulase components.

The existence of multiple endoglucanase and exoglucanase forms may be due to several factors. These include the existence of multiple genes, post-translational and proteolytic modifications of the gene products, variable cellulase composition, presence of other proteins, glycoproteins, and polysaccharides in the cellulase complex, and variable glycosylation of the cellulase components (Eveleigh, 1987). Moreover, the interaction of the enzyme with its environment may also affect its activity. Studies of cellulase synthesis in *Trichoderma spp.* have shown that the induction, expression, and secretion of cellulase are closely associated processes (Sternberg and Mandels, 1979).

In general, the endoglucanase and exoglucanase activities of fungal cellulases are extracellular, whereas β-glucosidase activity seems to be cell-associated (Eveleigh, 1987). As a result, cell-free broths of fungi have low β-glucosidase activity. In order to address this deficiency, hypersecretive mutants have been isolated. Such a mutant of *Penicillium funicusolum*, when cultivated on 3% rice bran at pH 4.5, yielded β-glucosidase activity as high as 30,000-36,000 IU/L (Lachke et al., 1983).

Mechanism of Enzymatic Action

The three components of fungal cellulases (endoglucanase, exoglucanase, and β-glucosidase) are believed to function synergistically. A generally accepted enzymatic mechanism distinguishes their actions (Eveleigh, 1987): Endoglucanases initiate attack by randomly cleaving β-1,4-glucosidic bonds and thus creating shorter-length cellulose chains; neighboring exoglucanases start degrading these chains at the nonreducing termini, thus generating cellobiose and glucose residues; finally, β-glucosidases cleave cellobiose to form glucose units. However, as cellulose hydrolysis proceeds, its rate decreases and eventually ceases before all the substrate is hydrolyzed.

Based on observations that amorphous cellulose is more readily hydrolyzed by cellulase than crystalline cellulose, it has been postulated that as the hydrolysis progresses, the cellulosic biomass becomes enriched in crystalline cellulose, and hence the hydrolysis rate diminishes. Other factors that may also be contributing to the decrease in the hydrolysis rate include enzyme adsorptive loss to lignin, thermal, mechanical, and/or chemical enzyme deactivation, and enzyme inhibition by the hydrolysis products cellobiose and glucose). Exoglucanases are primarily inhibited by cellobiose, whereas glucose inhibits mainly β-glucosidase. The SSF process circumvents the inhibition problems to a significant extent by allowing the simultaneous consumption of glucose by the employed fermentative microorganism.

Although useful, the outlined enzymatic mechanism model cannot account for the various specificities of the multiple forms of endoglucanase and exoglucanase and their interactions. The case of *T. reesei* cellulase components (purified) illustrates the shortcomings of the model: Optimal synergism between CBH II and EG II or EG I has been observed at a ratio of 95:1, unlike a 1:1 optimal ratio reported for CBH I and EG I, as well as CBH I and EG II (Henrissat et al., 1985). Interestingly, even cooperation between CBH I and CBH II has been described (Fāgerstam and Pettersson, 1980), with an optimum at a ratio of 1:4 (Henrissat et al., 1985). It should be noted, however, that the degree of cooperation among the components varied from one substrate to another.

The rate and extent of cellulose hydrolysis catalyzed by cellulase can be considered a function of primarily the concentration, composition, and condition of the substrate and the concentration, composition, and stability of cellulase (Philippidis, 1990). The activity of cellulase varies widely among the various cellulosic substrates. The crystallinity (Lee and Fan, 1983) and surface area (Grethlein, 1985; Converse et al., 1990) seem to be the two major characteristics of cellulose that affect its susceptibility to degradation by cellulase. Dyed substrates, such as Ostazin Brilliant Red H-3B-hydroxyethyl cellulose (Farkas et al., 1985) and Remazol Brilliant Blue R cellulose (Holtzapple et al., 1984) have been used for enzyme detection and activity measurement with increased sensitivity. Carboxymethyl cellulose (CMC) serves as substrate to endoglucanases exclusively, whereas the β-glucosidase activity of cellulase is determined by hydrolysis of p-nitrophenyl-β-glucopyranoside. The overall activity of cellulase is measured using filter paper cellulose as substrate (Mandels et al., 1976). It is expressed in international units (IU), where one IU of cellulase activity on filter paper is the amount of enzyme that yields 1 μmol of equivalent glucose per minute. This activity unit is used throughout this report to describe the cellulase volumetric production rate (productivity) in IU/L/h, the cellulase yield on substrate in IU/g, and the cellulase concentration in IU/L.

ENZYME PRODUCTION

The cost of substrate utilized for cellulase synthesis plays a crucial role in determining the economics of enzyme production. It is therefore imperative to identify inexpensive substrates that lead to high levels of cellulase productivity and yield. Several substrates have been evaluated as carbon sources for the production of cellulase, both insoluble (cellulose, hemicellulose, agricultural residues, whey, corn stover, wheat straw, steam-exploded wood, rice bran) and soluble (lactose, cellobiose, xylose) substances. The identity of the carbon source may affect not only the production of cellulase but also its composition. It has been reported that when *T. reesei* grows on cellulose or lactose, the composition of the cellulase complex is better balanced with regard to its overall activity than with other carbon sources, such as sophorose (Sternberg and Mandels, 1979).

Table 1 at the end of the report summarizes representative data from various cellulase production processes, ranging from shake flask experiments to 3,000-L fermentations. The operational conditions of each process and the corresponding cellulase productivities and concentrations are reported in the table to give an overview of the current status of the cellulase production technology. In addition, using the substrate and cellulase concentration data, the yields of enzyme with respect to the carbon source or substrate (in the case of lignocellulosic biomass with unspecified composition) used have been calculated and included in Table 1.

Substrate selection and media optimization

Cellulase production and activity are influenced by several factors, such as the carbon, nitrogen, and phosphorus sources, the ratio of carbon to nitrogen provided, trace elements, pH, and aeration rate (Mandels and Reese, 1957; Tangnu et al., 1981; Shinde and Gangawane, 1986; Brown et al., 1987b). Soluble carbohydrates promote faster cell growth and therefore decrease the fermentation time needed for cellulase production. Lactose has been reported to enhance cellulase productivity and yield, when compared with cellulose (Frein, 1986), presumably due to the higher cell growth rate achieved in the presence of the soluble substrate (lactose). However, soluble sugars are expensive, thus raising the cost of the enzyme. For this reason, less expensive cellulosic substrates have been examined. Among them, steam-exploded aspen wood is an attractive choice because of its good digestibility. Unfortunately, when aspen wood was used as the sole carbon source of *T. reesei* RUT-C30, cellulase activity was low (1,600 IU/L) and the enzyme was deficient in exoglucanase and β-glucosidase (Khan and Lamb, 1984). Substituting 10-20% of the wood (in equivalent cellulose) by pure cellulose brought the cellulase production and composition to the levels of cellulase synthesized from pure cellulose. Table 2 illustrates the effect of this substitution on cellulase production.

Numerous media have been developed and evaluated for use in cellulase production. A typical medium composition is shown in Table 3. Some metallic ions (Ca^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+}) have been found necessary for cellulase synthesis, although they did not enhance cell growth; it was postulated that they might be affecting enzyme secretion or preventing an inducer molecule from diffusing out of the cell (Mandels and Reese, 1957). The presence of Tween-80 at a concentration of 0.1-0.2 ml/L reportedly results in enhanced cellulase production by increasing the permeability of the cell membrane and thus improving enzyme secretion (Tangnu et al., 1981). In general, *T. reesei* grows well in defined media containing lactose, ammonium salts, and minerals (Sternberg and Dorval, 1979), although it grows faster in rich (undefined) media of fructose or glucose as carbon source and peptone as nitrogen source (Ryu and Mandels, 1980). The use of amino acids (Ryu and Mandels, 1980) and yeast extract (Meyer and Humphrey, 1982) as nitrogen sources seems to stimulate cell growth and cellulase synthesis. The maximal specific growth rate of *T. reesei* mutants cultivated in defined media reportedly ranges from 0.1 to 0.25 h^{-1}, depending on the identity of the carbon and nitrogen sources employed (Ryu and Mandels, 1980). Sorbose acts as an inducer of cellulase synthesis. When *T. reesei* was cultivated on 10 g/L xylose, cellulase production was half of that observed with 10 g/L of Solka Floc cellulose (Schaffner and Toledo, 1991). Supplementation of the xylose with 3 g/L sorbose doubled enzyme production without affecting cell growth.

In a recent study, the importance of media optimization was demonstrated. Various nitrogen and carbon sources were examined for the production of cellulase by the fungus *Penicillium pinophilum* (Brown et al., 1987b). The nitrogen sources under consideration were casein

Table 2. Cellulase production by *Trichoderma reesei* RUT-C30 in peptone medium containing steam-exploded aspen wood and various amounts of pure Solka Floc cellulose (compiled from Khan and Lamb, 1984).[1]

CELLULOSIC SUBSTRATE[2]	ENZYMATIC ACTIVITY (IU/ml)[3]			
	CELLULASE	EXO	ENDO	β-G
WOOD (Aspen)	1.6	1.0	7.7	0.2
WOOD+CELLULOSE (95/5)	3.1	2.2	7.9	0.3
WOOD+CELLULOSE (90/10)	3.6	2.5	8.2	0.4
WOOD+CELLULOSE (80/20)	3.9	2.6	8.7	0.4
CELLULOSE	4.1	2.7	9.2	0.5

[1] Exo, Endo, and β-G are the exoglucanase, endoglucanase, and β-glucosidase components of cellulase.

[2] The numbers in parenthesis represent the percentage, on a weight basis, of each component in the mixed substrate.

[3] Although all activities are expressed in International Units (IU) per ml, the substrate of reference for each cellulase component is different (see Khan and Lamb, 1984). Therefore, for meaningful comparisons the table should be read vertically, not horizontally.

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hydrolyzate, peptone, yeast extract, corn steep liquor, urea, and inorganic nitrogen salts. Among them, corn steep liquor yielded the highest cellulase concentration, more than 2,000 IU/L, whereas $NaNO_3$ was the source that led to the highest cellulase specific activity, 0.78 IU/mg protein, and $NH_4H_2PO_4$ the source that resulted in the lowest cellulase concentration, 400 IU/L. The carbon source was 10 g/L hammer-milled barley straw. Hence, use of the optimal nitrogen source in that study resulted in a five-fold improvement in enzyme production. In another study, however, the use of rich nitrogen sources (yeast extract and peptone) had no effect on enzyme concentration and productivity (Watson et al., 1984). These two nitrogen sources only reduced slightly the lag phase of cell growth. It has been reported that nitrogen supplied in the form of $Ca(NO_3)_2$ yielded the highest cell mass and cellulase concentrations for the fungus *Phoma Herbarum* cultivated on 10 g/L cellulase (Shinde and Gangawane, 1986). The use of urea as nitrogen source has also been suggested (Tangnu et al., 1981), since it increases cellulase, thus shortening the duration of the cellulase fermentation (Table 3).

A comparative study has been performed in which the carbon sources tested were Solka Floc cellulose, avicel cellulose, hammer-milled barley straw, ball-milled barley straw, wheat bran, and combinations of these (Brown et al, 1987b). Avicel cellulose (10 g/L), the best source, yielded

Table 3. Typical medium composition used for cellulase production; undefined media usually include 1 g/L of peptone, yeast extract, or corn steep liquor (compiled from Watson et al., 1984).

COMPONENT	CONCENTRATION
Cellulose	10.0 g/L (variable)
$(NH_4)_2SO_4$	1.4 g/L
KH_2PO_4	2.0 g/L
$MgSO_4.7H_2O$	0.3 g/L
$CaCl_2.2H_2O$	0.4 g/L
$FeSO_4.7H_2O$	9.2 mg/L
$MnSO_4.H_2O$	2.0 mg/L
$ZnSO_4.7H_2O$	2.7 mg/L
$CoCl_2.6H_2O$	2.0 mg/L
Urea (optional)	0.3 g/L
Tween-80 (optional)	0.1-0.2 ml/L

almost 4,000 IU/L, a five-fold improvement in enzyme yield over wheat bran. In this case, the specific activity of cellulase was 0.90 IU/mg, slightly lower than that of cellulase produced from a mixture of 5 g/L avicel cellulose and 5 g/L ball-milled straw (1.02 IU/mg); a mixture of corn steep liquor and inorganic salts was used as nitrogen source. Enzyme yield also depends on substrate concentration. When the substrate (a cellulose and ball-milled straw mixture) concentration was raised from 15 to 60 g/L, the *P. pinophilum* cellulase concentration at the end of three days of cultivation increased five-fold (Brown et al., 1987b). Batch fermentation of a mixture of 30 g/L avicel cellulose and 30 g/L hammer-milled straw yielded close to 10,000 IU/L cellulase in 72 hours at a production rate of 137 IU/L/h (Brown et al., 1987b). That represents a significant cellulase yield of 167 IU/g substrate. In the above study, the pH of the cultivation medium was left uncontrolled in the 3.5-6.0 range and the temperature was set at 35°C (Table 1). Proportional increase in cellulase concentration has been reported for *T. reesei* up to 50 g/L of cellulose, reaching a maximum of 8,000 IU/L at a production rate of 55 IU/L/h (Hendy et al., 1984). Above that cellulose concentration, however, little further increase in cellulase productivity was observed, as the viscosity of the medium increased significantly and the cell growth rate slowed down.

The effect of lignin on *T. reesei* cellulase production and activity has also been studied (Vohra et al., 1980). Although lignin and components of lignin (vanillin, protocatechuic acid, and verulic acid) had no effect on enzyme activity, in general they influenced negatively the growth of *T. reesei* and the production of cellulase components. It is worth mentioning that addition of 0.3 g/L conifer lignin to the growth medium resulted in a two-fold decrease in cell mass, eight-fold decrease in exoglucanase activity, and five-fold decrease in endoglucanase and β-glucosidase activity. Supplementation of 0.5 g/L lignin led to complete inhibition of cellulase synthesis. The effect of lignin components varied among the cellulase components. This potential effect of lignin on cellulase production should be investigated, since it is not known whether the lignin present in lignocellulosic biomass will have a similar effect on cellulase production as pure lignin.

Fermentation conditions for cellulase production

As shown in Table 1, the optimal temperature for fungal cellulase production is around 28°C, since these fungi are mesophilic organisms. In contrast, thermophilic bacteria prefer temperatures around 55°C. More variability is found in the optimal pH value, which ranges between 3.2 and 6.8. In a study of the pH effect on cellulase production, although a pH of 5.0 enhanced overall cellulase synthesis, the productivity of a particular cellulase component, β-glucosidase, was maximal at a pH value of 6.0 (Tangnu et al., 1981). It seems that by manipulating the temperature and pH conditions of the fermentation, the composition of cellulase can be influenced. Interestingly, in the case of a *Penicillium pinophilum* mutant grown on 60 g/L of straw or cellulose, maximal cellulase production (about 6,000 IU/L) was obtained when the pH was allowed to fluctuate naturally between 3.5 and 5.0 (Brown et al., 1987a).

Oxygen is another element that plays an important role in cellulase production. Although the oxygen uptake rate during the stage of cell growth is 4-fold higher than during enzyme synthesis, the provision of adequate oxygen during this latter stage is imperative for enzyme synthesis (Ryu and Mandels, 1980). A minimal dissolved oxygen concentration of 20% of the saturation level reportedly satisfies the cellulase synthesis needs (Brown et al., 1987a; Robison, 1984; Tangnu et al., 1981). Associated with oxygen is the formation of foam during the fermentation for cellulase synthesis. In order to circumvent the problem, various antifoam agents have been employed (Tangnu et al., 1981; Meyer and Humphrey, 1982).

As noted earlier, *T. reesei* cellulases have low β-glucosidase activity, which limits their ability to convert cellulose to glucose. In order to remedy this disadvantage, co-cultivation of *T. reesei* with high β-glucosidase producing organisms has been attempted, such as *Aspergillus niger* (Knappert et al., 1980), *Aspergillus phoenicis* (Allen and Sternberg, 1980), *Schizophyllum commune* (Desrochers et al., 1981), and even immobilized *Alcaligenes faecalis* (Wheatley and Phillips, 1983). The β-glucosidase synthesized by these organisms complemented the high endoglucanase and exoglucanase activities of *T. reesei* cellulases. In the case of immobilized enzyme, however, external and internal diffusion limitations were reported for the β-glucosidase activity. As an alternative to β-glucosidase supplementation, cellobiose-fermenting organisms, such as *Hansenula spp.* (Cavazzoni and Adami, 1987) or *Streptomyces spp.* (Moldoveanu and Kluepfel, 1983) could be used in conjunction with *T. reesei*. The use of mixed cultures successfully complemented the high cellulase production levels of *Trichoderma harzianum* with the high β-glucosidase activity of *Aspergillus ustus* (Macris et al., 1985). The cultivation was carried out on cotton fiber and wheat straw cellulose.

Identification of improved cellulase-producing organisms

In an effort to reduce the production cost of cellulase, hypersynthesizing mutant strains of cellulolytic microorganisms have been created and identified using UV light and/or mutagenic agents. As a result, enzyme productivities as high as 427 IU/L/h have been attained during fed-batch fermentations of *T. reesei* (Watson et al., 1984). Enzyme yields reaching 288 IU/g cellulose have been reported for a batch cultivation of the same organism (Table 1). A mutant of *T.reesei* QM 9414, morphologically different from the parent strain (higher branching degree), when cultivated in a peptone medium containing 10 g/L cellulose, produced two-fold more cellulase than the parent strain (Farkas et al., 1981). Furthermore, the cellulase of another mutant had a three-fold higher β-glucosidase specific activity than the parent strain. Using mutants of *T. reesei* grown for 14 days on 6% roll-milled cotton in a 10-liter pH controlled fermentor, the enzyme production rate reached 80 IU/L/h in a batch system and 7 IU/g cell mass/h in a continuous culture set-up (Ryu and Mandels, 1980). Moreover, mutant strains of *T. reesei* have been isolated with cellulase productivities two to six-fold higher than that of the wild strain, without any effect on enzyme composition (30% endoglucanase, 70% exoglucanase, and less than 1% β-glucosidase) (Shoemaker et al., 1981; Creese, 1983). Mutagenesis of the *T. reesei* strain QM 9414 with UV light and nitrosoguanidine (a mutagen) yielded a hyperproducing and catabolite repression-resistant mutant (Ghosh et al., 1982). Cultivation of this mutant in peptone media containing 6% Solka Floc cellulose in a 150-liter batch fermentor at 28°C and pH of 3.2 yielded 11,000 IU/L of cellulase at an enzyme production rate of 57 IU/L/h, a 90% improvement over the parent strain.

The method of mutation and selection has been also used to improve strains of cellulolytic bacteria, such as *T. fusca* (Meyer and Humphrey, 1982). Furthermore, recombinant DNA techniques have been employed to develop cellulase overproducing systems. *Escherichia coli* is a popular host for foreign proteins, but in the case of cellulase components the inability of the bacterium to glycosylate the gene products may result in the formation of less active or inactive proteins. Glycosylation of cellulase, although not fully comprehended, seems to play an important role in substrate recognition and enzyme stability.

Cellulases, in general, have low specific activities (typically, 100-fold less than amylases). Thus, research is being conducted to increase cellulase activity via strain mutation to produce more active enzymes, site-specific mutagenesis to improve the catalytic activity of the active site, and isolation of novel microorganisms (Eveleigh, 1987). A cellulase isolated from the anaerobic fungus *Neocallimastix frontalis* was several times more active than the *T. reesei* Rut-C 30 cellulase (Wood et al., 1986). Unfortunately, these optimization methods usually do not always lead to the same extent of improvement for the endoglucanase and exoglucanase activities of cellulase. More efficient mutant selection methods and improved enzyme characterization techniques are needed to help optimize cellulase production. The development of monoclonal antibodies specific for each cellulase component (currently underway by a SERI subcontractor) will offer insight into the synergism between endoglucanases and exoglucanases and elucidate the CBH to EG ratio that leads to optimal cellulose hydrolysis rates.

Thermostable cellulases are of particular interest, because of their longer activity, stability, and resistance against chemicals. The use of thermophilic organisms (primarily bacteria) offers several advantages: Higher growth and reaction rates at elevated temperatures, increased mass transfer rates, low cooling requirements for the fermentation, and lower contamination risk. Disadvantages include more expensive construction materials, water evaporation, and low oxygen solubility in the fermentation media at high temperatures.

Several thermophilic bacteria (50-60°C) secrete thermostable cellulases resistant to proteolysis and chemical and mechanical denaturation. As an example, *Acidothermus cellulolyticus* cellulase has temperature and pH optima of 55°C and 5.2, respectively (Mohagheghi, 1986). When grown on cellulose or cellobiose, *A. cellulolyticus* produces the enzyme at the late exponential and early stationary phase of its growth, similarly to *T. reesei* (Ryu and Mandels, 1980), with D-cellobiose and D-xylose acting as inducers (Shiang et al, 1990a). Cellulase synthesis is apparently inducible by soluble derivatives of cellulose (e.g. cellobiose) and other low molecular weight carbohydrates, such as lactose, sophorose, and xylose (Shiang et al, 1990a). It is also subject to catabolite repression by readily metabolizable sugars, such as glucose. That repression is relieved by addition of exogenous cAMP (Shiang and Linden, 1990b).

The main drawback in the use of bacteria is their low cellulase productivities, as exemplified in Table 4. In comparison, *T. reesei* RUT-C30 is the organism with the highest productivity reported to date, 427 IU/L/h, when cultivated on 232 g/L hardwood ball-milled cellulose in a fed-batch mode (Watson et al., 1984). The same is true for cellulase yield (Table 1). In an effort to remedy this disadvantage, various compounds in addition to cellulose have been screened for their ability to induce bacterial cellulase synthesis. The thermophile *A. cellulolyticus* has served as a potential source of thermostable cellulases, especially when cultivated in the presence of cellulose. As noted earlier, 15 g/L Solka Floc cellulose increased three-fold the cellulase concentration in the fermentation broth, compared with cultivation in cellobiose (Shiang et al., 1990a). A recent literature survey has found that the bacteria *Cellulomonas fimi, Thermomonospora fusca, Pseudomonas fluorescens* and *cellulosa, Acetovibrio cellulolyticus,* and *Acidothermus cellulolyticus* synthesize highly active endoglucanases and exoglucanases and therefore deserve closer attention (Himmel, 1990).

ENGINEERING ASPECTS OF CELLULASE PRODUCTION

During the batch cultivation of *T. reesei* for production of cellulase (e.g. Ryu et al., 1979; Meyer and Humphrey, 1982), two stages can be identified: First, cell growth is observed, followed after a lag period by enzyme synthesis and secretion, which increases to significant as cell growth begins entering the stationary phase. The two stages of cell growth and cellulase synthesis exhibit different temperature and pH optima. Growth is favored by temperatures in the range of 32-35°C, whereas enzyme production occurs optimally at 25-28°C (Ryu and Mandels, 1980). Similarly, cell growth is optimal at a pH of approximately 4.0, while cellulase synthesis needs a pH of 3.0. Other researchers have reported different optima (Table 1).

The different requirements in the two stages of cellulase production can serve as a guide for developing a strategy towards optimization of enzyme productivity: In batch or fed-batch mode, the optimal profiles of pH and temperature should be applied to the system, whereas in continuous operations a two-stage process would be desirable, where the first stage will bring cell concentration to high levels and the second stage will allow solely cellulase synthesis to take place.

Table 4. Cellulase production by thermophilic bacteria and mesophilic fungi (*T. reesei*) grown on cellulosic substrates (compiled from Margaritis and Merchant, 1986, and Shiang et al., 1990a).

ORGANISM	SUBSTRATE	ENZYME CONC. (IU/L)	PRODUCTION RATE (IU/L/h)	FERM. TIME (h)
Thermomonospora fusca	Avicel	150	5.1	29
Thermomonospora curvata	Cellulose	100	1.4	72
Clostridium thermocellum	Solka Floc	140	1.9	72
Acidothermus cellulolyticus	Solka Floc	105	1.5	70
Thielavia terrestris	Solka Floc	110	3.4	32
Trichoderma reesei QM 9414	Solka Floc	2,600	21.7	120
Trichoderma reesei QM 6a	Solka Floc	5,000	15.0	333
Trichoderma reesei L27	Avicel	18,000	93.7	192

Batch cellulase production

Batch is the most common operational mode of cellulase production, apparently because it is the easiest to start up and control. Depending on the organism, cellulase concentrations as high as 15,000 IU/L and enzyme yields of 288 IU/g cellulose have been achieved (Table 1). The high enzyme activity was obtained with the mesophilic fungus *T. reesei* cultivated in the presence of 60 g/L roll-milled cotton cellulose (Ryu and Mandels, 1980). A pilot plant study of *T. reesei* CL-847 cellulase production was carried out in a 3,000 L fermentor using 60 g/L of lactose as carbon source and 1 g/L of yeast extract as nitrogen source (Warzywoda et al., 1983). Cellulose was also provided at a concentration of 5 g/L to induce cellulase production. After eight days of cultivation, the cellulase concentration reached 10,500 IU/L with a satisfactory overall enzyme yield of 162 IU/g carbon source. The use in SSF of whole *T. reesei* L27 fermentation broth, as a source of cellulase, has proved to be successful (Schell et al., 1990). The study showed that whole broth exhibits higher cellulase activity than the culture filtrate, thus leading to higher ethanol yields in the SSF process. The improvement in enzyme activity is due to the additional cellulase present in the cell mass of the mycelia. In addition to providing higher enzyme activity, the use of whole broth eliminates the need for separation of the solids in the effluent of the cellulase production fermentor.

Although batch fermentations lead to high cellulase concentrations and yields, their productivities usually suffer. In a *T. reesei* study, the productivity did not exceed 45 IU/L/h when cultivated on cotton cellulose (Ryu and Mandels, 1980), a value relatively small compared to the productivities of fed-batch and continuous operations (Table 1). Another fungus, *P. pinophilum*, yielded cellulase at a rate of 137 IU/L/h, but the enzyme concentration was only 10,000 IU/L (Brown et al., 1987b).

The feasibility of cellulase production using inexpensive lignocellulosic biomass as carbon source was investigated in a batch 5-L fermentor with *T. reesei* MCG-77 (Doppelbauer et al., 1987). Several substrates were examined, ranging from municipal waste to pretreated wheat straw. The results are shown in Table 5. Bleached sulfite pulp was used as a reference substrate, since it is a good but relatively more expensive material. Interestingly, considerable production potential was exhibited by the residual wheat straw from hydrolysis with *T. reesei* cellulase (10 IU/g for 24 hours at 50°C); the enzyme productivity was 31 IU/L/h and the concentration 1,900 IU/L. Similar results were obtained with NaOH-treated municipal waste and steam-treated straw, which yielded cellulase at rates of 24 and 31 IU/L/h, respectively. Waste paper yielded half of the enzyme activity per unit amount of carbohydrate content compared to municipal waste. Steam treatment improved almost two-fold the yield of cellulase from wheat straw. By increasing the straw concentration from 20 to 50 g/L, the cellulase (filter paper) and β-glucosidase activities doubled to 3,800 and 1,950 IU/L, respectively. Controlling the pH at 6.0 was beneficial to cellulase production. The data of this study indicate that inexpensive lignocellulosic biomass, such as waste paper and fresh or hydrolyzed wheat straw, can support production of cellulase at considerable rates.

In another study of lignocellulosic materials, steam-exploded aspen wood was evaluated as a substrate for cellulase production by *T. reesei* RUT-C30, and its performance was compared to that of pure Solka Floc cellulose (San Martin et al., 1986). Batch cultivation of the fungus on 50 g/L of aspen yielded 4,500 IU/L of cellulase at a considerable rate of 50 IU/L/h. The productivity was similar to that on Solka Floc, but the enzyme concentration was 50% lower. By increasing the aspen concentration to 90 g/L, the enzyme activity increased to 7,200 IU/L, although the production rate decreased further to 39 IU/L/h. These results were similar to those obtained with 75 g/L Solka Floc and suggest that cellulase synthesis using pretreated wood, a promising substrate for ethanol production, is a feasible scheme.

The batch production of cellulase by the thermophilic bacterium *A. cellulolyticus* has also been investigated (Shiang et al., 1990a). Among various carbon sources tested, this thermophile synthesized cellulase only when it grew on cellulose, xylose, and cellobiose, with specific growth rates of 0.10, 0.14, and 0.18 h^{-1}, respectively. When cultivated in yeast extract supplemented with salts, minerals, and 2.5-10 g/L cellobiose as its carbon source at 55°C and pH 5.2, *A. cellulolyticus* produced cellulase at a rate of 0.92-2.49 IU/L/h and a final concentration of 21-69 IU/L. Both the productivity and the concentration of cellulase increased proportionally to the cellobiose concentration. Supplementation of the media with avicel cellulose (5-16 g/L) or Solka Floc cellulose (15 g/L) increased further the enzyme concentration up to a maximum of 105 IU/L, although the presence of cellulose slowed down the growth rate of the organism. Solka Floc seemed to have a greater effect on enzyme synthesis than avicel. Unfortunately, neither of the celluloses had any impact on cellulase productivity.

Table 5. Cellulase production on various lignocellulosic substrates in a 5-L batch fermentation (compiled from Doppelbauer et al., 1987).

CELLULOSIC SUBSTRATE	SUBSTRATE CONCENTR. (g/L)	ENZYME CONC. (IU/ml)	PRODU-CTIVITY (IU/L/h)	ENZYME YIELD (IU/g)[1]
Spruce sulfite pulp	20	3.7	61	185
Wheat straw (untreated)	20	1.1	13	79
Wheat straw (steamed)	20	1.9	31	151
Wheat straw (NaOH treated)	20	2.0	25	133
Wheat straw (8-day stored)	20	1.6	27	97
Wheat straw (hydrolyzed)	30	2.4	31	147
Printed newspaper	30	0.7	19	48
Mixed waste paper	35	1.1	17	64
Municipal waste	30	2.5	24	130

[1] Enzyme yield is expressed in IU of cellulase per gram of carbohydrate (cellulose and hemicellulose) content of the substrate

Fed-batch cellulase production

Another mode of *T. reesei* cultivation is the fed-batch process. Its main advantages, when compared with a batch or continuous operation, are the higher cellulase productivity and concentration, as well as the higher enzyme yield per unit mass of substrate utilized. This is primarily due to the more efficient use of the substrate achieved in a fed-batch system by manipulating the feed rates of media and substrate. Using a mixed substrate feed of cellulose (20 g/L) and xylose (30 g/L) and feed rates of 15 g/L/day and 5 g/L/day, respectively, an enzyme concentration of 12,500 IU/L and a productivity of 45.4 IU/L/h were attained (Mohagheghi et al., 1990). In that study it was observed that substitution of up to 25% (w/w) of the cellulose feed by xylose improved enzyme production. The rationale behind this substitution is to enhance the low growth rate of the fungus on cellulose by using a soluble carbohydrate (xylose). Incidentally, xylose is a convenient substrate for a biomass-to-ethanol conversion process, since it is readily available from the hydrolysis of hemicellulose, which occurs during the biomass pretreatment step. Furthermore, the amount of cellulose spared from consumption in the cellulase fermentor will be available for ethanol production in the SSF process.

In a comparative study of a batch, fed-batch, one-stage continuous, and two-stage continuous production of cellulase from *T. reesei* RUT-C30 using Solka Floc cellulose, the fed-batch operation was reportedly superior to the other modes with regard to the concentration, yield, and productivity of the enzyme (Hendy et al., 1984). Cellulase was added whenever a decrease in cell growth rate was detected. Cellulase concentration was found to be proportional to the substrate feeding rate at the expense, however, of enzyme productivity. After 8.3 days of cultivation in 100 g/L Solka Floc cellulose, the cellulase concentration reached 22,400 IU/L at an average production rate of 247 IU/L/h, and the corresponding enzyme yield was high, 224 IU/g cellulose. In a fed-batch study with the same organism but 232 g/L of ball-milled cellulose, the productivity achieved was 427 IU/L/h and the concentration 57,000 IU/L (Watson et. al., 1984). These values are the highest ones reported to date. In that study, the temperature was set at 27°C during the first 42 hours of operation in order to promote cell growth and then was switched to 25°C to enhance cellulase production.

Using steam-exploded aspen wood, the fed-batch production of cellulase was studied with *T. reesei* RUT-C30 (San Martin et al., 1986). When aspen was provided at a feed rate of 2 g/h, cellulase concentration reached 15,000 IU/L at a production rate of 48 IU/L/h; the total wood concentration was 150 g/L. It was postulated that the accumulation of steam explosion by-products and lignin in the fermentor decreased the performance of the lignocellulosic biomass.

Continuous cellulase production

Using a two-stage continuous system, researchers have investigated the production of cellulase by *T. reesei* MCG-77 cultivated in a defined medium of lactose (50-100 g/L), ammonium sulphate, and mineral salts in 15-liter fermentors (Ryu et al., 1979). Two different sets of conditions were used for the two stages of cellulase production: (a) 32°C, pH 4.5, 600-800 rpm agitation, and 0.3-0.5 vvm aeration for cell growth, and (b) 28°C, pH 3.5, 400-600 rpm agitation, and 0.2-0.3 vvm aeration for cellulase synthesis. A silicone agent was used for foam control. Variables and parameters of the two stages are summarized in Table 6. The specific flow rates of the carbon, nitrogen, and oxygen sources were used as process control variables to maximize enzyme productivity, taking into consideration that the three nutrients were utilized for biomass formation, maintenance requirements, and cellulase biosynthesis. The specific growth rate maintained during cell growth was 0.1 h^{-1}. In the second stage maximal cellulase productivity was obtained at a specific growth rate value of approximately zero, while the dilution rate was set at about 0.03 h^{-1}. Enzyme productivity decreased at higher dilution rates. At steady state, the cell mass concentration was maintained around 10 g/L, bringing the enzyme productivity of the continuous process to 90 IU/L/h (8 IU/g cell mass/h).

Continuous cellulase production has also been studied using *T. reesei* RUT-C30 in a one-stage and two-stage operation (Hendy et al., 1984). In the latter case, the working volume of the first stage was half of the volume of the second stage; both stages were operated at the same conditions (28°C and pH 5.0). At a cellulose feed concentration of 20 g/L, the one-stage system yielded 2,100 IU/L of cellulase at a rate of 97 IU/L/h and a yield of 105 IU/g, whereas the two-stage operation resulted in a higher cellulase concentration (3,300 IU/L) and enzyme yield (165 IU/g), but significantly lower productivity (58 IU/L/h). In the single stage process, increasing the dilution rate beyond 0.046 h^{-1} led to lower enzyme productivities. In the two-stage system, the optimal productivity was achieved at a dilution rate of 0.017 h^{-1}; productivity decreased at higher dilution

Table 6. Characteristics of a two-stage continuous cultivation of *Trichoderma reesei* for the production of cellulase (compiled from Ryu et al., 1979).

VARIABLE/PARAMETER[1]	STAGE 1 BIOREACTOR	STAGE 2 BIOREACTOR
F_C	100	16.0
F_O	128	32.0
F_N	8.0	1.0
m_C	10.0	10.0
m_O	27.2	27.2
m_N	0	0.9
Y_{XC}	1.1	1.1
Y_{XO}	1.0	1.0
Y_{XN}	12.5	16.6

[1] F_j is the specific feed rate of nutrient j (mg/g cell mass/h); m_j is the maintenance coefficient of nutrient j (mg/g cell mass/h); and Y_{Xj} is the yield coefficient of cell mass with respect to nutrient j (g cell mass/g nutrient).

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rates. In another study, continuous one-stage cellulase production by the thermophilic Thermomonospora sp. exhibited its highest productivity (39 IU/L/h) at a dilution rate of 0.125 h^{-1} (Meyer and Humphrey, 1982). Cell mass concentration also peaked at the same dilution rate value.

Another means of improving cellulase productivity is by cell recycle. For the one-stage and two-stage continuous systems described earlier, cell recycle resulted in high productivities at high dilution rates, 0.064 and 0.027, respectively (Hendy et al., 1984). Operating at high dilution rates decreases the residence time of the cultivation and thus its cost.

RECOMMENDATIONS FOR FUTURE WORK

The cost of cellulase is a significant factor in the economics of the biomass-to-ethanol conversion technology (Figures 2). As a result, it has a direct impact on the cost of the produced ethanol (Figure 3). It is therefore necessary to minimize the cost of the enzyme. Cellulase can be either obtained from out-of-house suppliers or synthesized in house. The disadvantage of an outside supplier is the fluidity in the quality, availability, and cost of the enzyme. Even if the cost of cellulase purchased is more competitive, the ethanol technology we are developing cannot afford the risk of uncertain availability and quality. It is therefore recommended that research be carried out at SERI to develop an enzyme production process.

The key to producing inexpensive and highly active cellulase lies in a combination of critical factors: Improved enzyme quality, enhanced enzyme productivity and yield, prolonged enzyme utilization, and minimal cost of media and substrate. Hence, if some of these factors cannot be improved beyond a certain point, then optimization of the others may serve as a compensation.

Enhancement of cellulase quality

The ideal cellulase would have:

(1) Complete and balanced composition of endoglucanase, exoglucanase, and β-glucosidase activities to convert efficiently and to the maximal extent cellulose into glucose; this is especially desirable for the fungal cellulases that contain low β-glucosidase levels.

(2) High specific activity to minimize the amount of enzyme needed in the SSF process, thus alleviating the need for high enzyme productivity and reducing the size of both the cellulase and SSF fermentors.

(3) Resistance to inhibition by hydrolysis (cellobiose, glucose) and fermentation (ethanol) products in order to retain its activity during the course of SSF and reduce the duration of the fermentation.

(4) Resistance to thermal, physical, and chemical deactivation to perform under conditions that may accelerate the rate of SSF or increase the ethanol yield.

(5) Little affinity for lignin to minimize the adsorptive loss and inactivation of cellulase components.

(6) High activity against various forms of lignocellulosic biomass to accommodate the use of multiple substrates for ethanol production.

In the search for better cellulases with features that fall in the above categories, it is essential to examine all possible sources: Bacteria, fungi, yeasts. Cellulases from these microorganisms need to be screened according to strict assay procedures that remain to be established, so that the properties of all the enzymes are compared on a consistent basis.

Anaerobic microorganisms should also be studied; for instance, the cellulase from the anaerobic fungus *Neocallimastix frontalis* has higher specific activity than the *T. reesei* RUT-C30 cellulase (Wood et al., 1986). It should be emphasized that a significant advantage of employing an anaerobe is the elimination of the aeration and agitation needs during cellulase production, which improves greatly the economics of the process.

Enhancement of cellulase yield and productivity

Enzyme yield and productivity directly affects the cost of cellulase. Improved enzyme yield reflects a more efficient utilization of the carbon source with respect to cellulase synthesis and hence reduces the cost of the substrate. Enhanced enzyme productivity diminishes the duration of the cellulase cultivation and thus decreases the number of fermentors required for a certain enzyme output and the capital cost of the process. Higher cellulase yields and productivities can be achieved by:

(1) Using a hyperproducing strain
(2) Using an organism that grows fast and reaches high cell concentrations
(3) Improving the configuration or conditions of the fermentation

Certainly, fungi have exhibited the highest enzyme productivities to date: Fed-batch cultivation of *T. reesei* on ball-milled hardwood pulp cellulose yielded 427 IU/L/h at a cell mass concentration of 35.9 g/L (Watson et al., 1984). As Table 1 shows, the same is true for *T. reesei* enzyme yield, which has reportedly reached 288 IU/g cellulose in a 10 L cultivation. Such high production levels indicate that possibly even higher productivities and yields may be reached in the future. In contrast, bacterial cellulase production is low: The thermophile *Thermonospore fusca*, which is considered a good bacterial producer, synthesized in a continuous operation only 39 IU/L/h of cellulase at an enzyme yield of 31 IU/g cellulose (Meyer and Humphrey, 1982). However, bacteria can reach high cell concentrations, such as 100-200 g/L at high growth rates, when cultivated appropriately. Moreover, cellulases from thermophilic bacteria function and remain active at temperatures exceeding 45°C, where reaction rates are considerably faster than at 28°C, the optimal temperature for most fungal cellulases. These features in conjunction with genetic manipulations and high specific activities make bacteria an attractive source of cellulases that should be investigated. By the same token, thermophilic fungi need also to be examined.

Mutagenesis of cellulolytic organisms using UV light and/or chemical agents has greatly improved cellulase production, as the literature search has shown. Although tedious, this technique may prove to be a great asset, especially for enhancement of the productivity of an already promising organism.

The configuration of the fermentation set-up, the mode of operation (be it batch, fed-batch, or continuous), and the conditions of the fermentation offer opportunities for improvements in the engineering section of cellulase production. The initial lag phase, common to all fermentations, can be minimized by selecting the optimal age and size of inoculum. As indicated earlier, cellulase production consists of two phases, cell growth and enzyme synthesis. Since the optimal pH and temperature conditions of the two phases may differ, it will be advantageous to manipulate these two variables, first to maximize cell mass production and then to boost cellulase synthesis.

For a batch or fed-batch operation, the optimal pH and temperature profiles need to be determined a priori through study of the kinetics of each phase, in order to be applied to the cellulase fermentor. Alternatively, two fermentor systems could be used, where the first will serve for cell growth and the second for enzyme production; each one will be operated at the optimal regime of the corresponding phase. The main disadvantage of this option is the increase in capital cost, due to the larger number of vessels needed.

For a continuous operation it may be advantageous to use two stages, one for each phase, since it would not be feasible to apply pH and temperature profiles in a single unit without disturbing the steady state of the system. Since each stage will operate under optimal conditions, high enzyme productivities and yields are to be expected, as experimental results have shown (Ryu et al., 1979), although the two-stage operation reportedly has lower enzyme productivity and yield compared with the one-stage system (Hendy et al, 1984). It should be noted that achieving steady state in a multiple bioreactor system is not a trivial task, especially when the output of the first stage (cell concentration) affects the performance of the second stage (enzyme production rate proportional to cell concentration). In one study it took 75-100 hours before steady state was reached (Ryu et al., 1979). Finally, the potential benefits of cell recycle, operation at higher dilution rates and increased productivity, need to be taken into consideration.

The two-stage set up and the fed-batch operation are promising and should be further investigated with the use of real substrate (lignocellulosic biomass), instead of soluble carbohydrates (e.g. lactose) or costly pretreated biomass (e.g. ball-milled biomass). Waste paper, municipal waste, newspaper, and agricultural residues are other abundant potential substrates, since they have reportedly supported cellulase synthesis (Table 5). Needless to say, of course, that the kinetic behavior of the employed microorganism has to be understood, before a successful fermentation is run. Investigation of the cellulase production kinetics is a priority and should also be used as a source of information for the formulation of a mathematical model that simulates the production of cellulase. Such a model, after parameter calibration and validation, will be a useful tool in optimizing the process and addressing scale-up concerns.

Another issue of major importance is the choice of process configuration for the integrated biomass conversion process. It needs to be examined whether enzyme synthesis should be a separate unit from the SSF system. The kinetics and economics of direct microbial conversion (DMC), which employs microorganisms (e.g. *Clostridium thermocellum*) that simultaneously synthesize cellulase and ferment the sugars (Lynd et al.,, 1989) should be compared with the performance of the separate cellulase-SSF scheme. DMC reduces the capital cost of ethanol production, but the problems of low ethanol yields and fermentation by-product formation need to be addressed. In addition to DMC, the feasibility, both technical and economical, of carrying out a mixed culture of an anaerobic cellulase-synthesizing strain and an ethanol-producing fermentative organism deserves careful evaluation, since it will result in significant reduction of capital and operating costs.

Enhancement of cellulase utilization

Reduction of the cellulase cost can also be achieved by extending the half life of the enzyme. This requires that the activity and stability of the enzyme be prolonged. Then, cellulase can be used repeatedly, either through recycle or immobilized in a reactor, thus decreasing the needs of SSF for enzyme. However, cell or enzyme recycle may require a costly means of separation. Immobilization or attachment of the enzyme, enzyme components, or whole cells on an appropriate matrix may extent the activity and stability of cellulases. This benefit, however, usually comes at the expense of lower hydrolysis rates, due to external and internal diffusion limitations raised by the immobilizing or attachment matrix (e.g. Wheatley and Phillips, 1983). The limitations can be alleviated to some degree by reducing the particle size of the immobilized biocatalyst and/or increasing the stirring speed in the fermentor. It therefore needs to be carefully investigated whether the prolonged use of the enzyme , which results in lower enzyme cost, can compensate for the slower rates and the cost of immobilization or biocatalyst separation in the case of recycle.

Substrate selection and media optimization for cellulase production

The cost of the media and carbon source used for enzyme production plays a major role in the economics of cellulase production. This is also true for the SSF process. It becomes, therefore, imperative to search for media that are both inexpensive and available at quantities that will satisfy the projected needs of ethanol production at the national level.

Soluble carbohydrates, such as lactose, xylose, and cellobiose, are popular carbon sources at the bench scale level, because they allow fast growth of the cells and induce cellulase synthesis. Their high cost, however, makes them prohibitive for a large scale ethanol production operation. Lignocellulosic biomass, on the other hand, provides a considerably less expensive carbon source and cellulase inducer, which is and will be in the future available at large quantities. Furthermore, cellulose acts as an inducer of cellulase synthesis. The drawback in the use of cellulose is the slow growth rate of the organisms, because they first need to hydrolyze cellulose to glucose before they can assimilate it. As reported in the literature, this problem can be alleviated by supplementing cellulose with small amounts of soluble sugars (e.g. cellobiose, xylose) or ball-milled biomass to enhance cell growth. Unfortunately, as in the case of soluble sugars, preparation of ball-milled biomass is costly. The area of substrate pretreatment needs to be further studied. A pretreatment method that can inexpensively make the substrate readily digestible by cellulase will benefit both the cellulase production and the SSF process.

Similarly to the carbon source, the cost and availability of the medium employed in cellulase production are important factors. The medium includes nitrogen, phosphorus, magnesium, and calcium sources, as well as trace elements. Usually peptone, yeast extract, or corn steep liquor are provided as rich sources of nitrogen, vitamins, and trace elements (Table 1). However, the cost of these ingredients may be prohibitive for the economics of cellulase production. Hence, defined media, such as that shown in Table 3, need to be evaluated for their effect on cell growth and enzyme synthesis and improved. The use of pretreated lignocellulosic biomass as substrate for the cellulose production unit and the SSF process may provide some of the essential nutrients and therefore reduce the complexity and cost of the supplied medium. Another likely source of nutrients is the cell mass present in the effluent streams of the SSF and xylose fermentation units. Experimental work should be conducted to assess the nutritional value of that source. As previous studies have shown (Tangnu et al., 1981; Brown et al., 1987; Duff et al., 1987), media optimization can have a great impact on cellulase productivity. In our case, the criterion for medium and substrate selection is a combination of optimal enzyme productivity, adequate source availability, and low cost.

CONCLUSIONS

The purpose of this milestone report was to assess the current status of research in cellulase production through a thorough review of the pertinent literature, which exceeded 350 publications. The ultimate goal is to recommend research directions that need to be pursued at SERI in order to establish our in-house cellulase production technology, which will provide low-cost cellulase enzyme and integrate it with the other units of the biomass conversion process.

From the presented literature overview, it is evident that we first need to understand the kinetics of cell growth and enzyme synthesis, using the most promising currently available microorganism. *T. reesei* strains are recommended for the initial research phase, since they are the highest cellulase producers. However, the best bacterial candidates (after initial screening by enzymologists) should also be examined, because they can reach high cell concentrations. Such candidates are *Cellulomonas, Thermonospora, Pseudomonas, Acetovibrio, and Acidothermus spp.* Moreover, it is recommended that anaerobic fungi (e.g. *Neocallimastix frontalis*) be also studied, because of their high specific activity and compatibility with SSF, which would eliminate any aeration requirements for enzyme production. Because of time limitations, decisions about novel ideas and alternative proposals (e.g. the DMC method) should be made rationally and swiftly with a certain amount of experimental work.

The formulation of a simple, descriptive model will be of great importance, since the model will serve as tool in determining interactions among process variables and their effect on enzyme productivity. After reaching this point, we will be in a position to pursue the most crucial issues of cellulase production recommended in earlier paragraphs: Substrate selection and media optimization, comparative investigation of the various types of mode of operation and engineering aspects of the cellulase fermentation system to enhance cellulase productivity and yield, prolonged enzyme activity, and testing of alternative cellulase producers and alternative layouts of the integrated biomass conversion process. It is recommended that the technical feasibility and performance of a fed-batch and a continuous enzyme production process be examined carefully, in view of their high productivity potential and their compatibility with the other continuous unit operations of the integrated biomass-to-ethanol conversion process.

REFERENCES

1. Allen, A. and Sternberg, D. *Biotechnol. Bioeng. Symp.* **10**, 189-197 (1980).

2. Augustin, J., Zemek, J., Kuniak, L., and Fassatiova, O. *Folia Microbiol.* **26**, 14-18 (1981).

3. Berg, B. *Can. J. Microbiol.* **21**, 51-57 (1975).

4. Brown, J.A., Collin, S.A., and Wood, T.M. *Enzyme Microb. Technol.* **9**, 176-180 (1987a).

5. Brown, J.A., Collin, S.A., and Wood, T.M. *Enzyme Microb. Technol.* **9**, 355-360 (1987b).

6. Cavazzoni, V. and Adami, A. *Ann. Microbiol.* **37**, 127-133 (1987).

7. Converse, A.O., Ooshima, H., and Burns, D.S. *Appl. Biochem. Biotechnol.* **24**, 67-73 (1990).

8. Coughlan, M.P. *Biochem. Soc. Trans.* **13**, 405-406 (1985).

9. Creese, E. M.S. Dissertation, University of Western Ontario, London, Ontario (1983).

10. Desrochers, M., Jurasek, L., Paice, M.G. *Appl. Environ. Microbiol.* **41**, 222-228 (1981).

11. Doppelbauer, R., Esterbauer, H., Steiner, W., Lafferty, R.M., and Steinmüller, H. *Appl. Microbiol. Biotechnol.* **26**, 485-494 (1987).

12. Duff, S.J.B., Cooper, D.G., and Fuller, O.M. *Enzyme Microb. Technol.* **9**, 47-52 (1987).

13. Enari, T.M. and Markannen, P. *Adv. Biochem. Eng.* **5**, 1-24 (1977).

14. Eveleigh, D.E. *Phil. Trans. R. Soc. Lond.* **321**, 435-447 (1987).

15. Fägerstam, L.G., and Pettersson, L.S. *FEBS Lett.* **119**, 97-110 (1980).

16. Fan, L.T., Lee, Y.-H., and Beardmore, D.H. in *Advances in Biochemical Engineering*, Vol. 14, (ed. Fiechter, A.), Springer-Verlag, Berlin, 101-117 (1980).

17. Farkas, V., Labudova, I., Bauer, S., and Ferenczy, L. *Folia Microbiol.* **26**, 129-132 (1981).

18. Feldman, K.A., Lovett, J.S., and Tsao, G.T. *Enzyme Microb. Technol.* **10**, 262-272 (1988).

19. Frein, E.M. Ph.D. Thesis, Rutgers University, New Brunswick, NJ (1986).

20. Ghosh, V.K., Ghose, T.K., and Gopalkrishnan, K.S. *Biotechnol. Bioeng.* **24**, 241-243 (1982).

21. Grethlein, H.E. *Biotechnology* 3, 155-160 (1985).

22. Grohmann, K., Torget, R., and Himmel, M. *Biotechnol. Bioeng. Symp.* **15**, 59-80 (1985).

23. Hendy, N.A., Wilke, C.R., and Blanch, H.W. *Enzyme Microb. Technol.* **6**, 73-77 (1984).

24. Henrissat, B., Driguez, H., Viet, C., and Schülein, M. *Biotechnology* **3**, 722-726 (1985).

25. Himmel, M., SERI Milestone Report "Complete the Literature Review of Reported Cellulases" (1990).

26. Hoffman, R.M. and Wood, T.M. *Biotechnol. Bioeng.* **27**, 81-85 (1985).

27. Holtzapple, M.T., Caram, H.S., and Humphrey, A.E. *Biotechnol. Bioeng.* **26**:753-757 (1984).

28. Kalra, M.K. and Sandhu, D.K. *Acta Biotechnol.* **6**, 161-166 (1986).

29. Khan, A.W. and Lamb, K.A. *Biotechnol. Lett.* **6**, 663-666 (1984).

30. Knappert, D., Grethlein, H., and Converse, A. *Biotechnol. Bioeng.* **22**, 1449-1463 (1980).

31. Lee, Y.-H., and Fan, L.T. *Biotechnol. Bioeng.* **25**, 939-966 (1983).

32. Lachke, A.H., Bastawde, K.B., Powar, V.K., and Srinivasan, M.C. *Biotechnol. Lett.* **5**, 649-652 (1983).

33. Lynd, L. R., Grethlein, H.E., and Wolkin, R.H. *Appl. Environ. Microbiol.* **55**, 3131-3139 (1989).

34. Macris, B.J., Paspaliari, M., and Kekos, D. *Biotechnol. Lett.* **7**, 369-372 (1985).

35. Mandels, M. and Reese, E.T. *J. Bacteriol.* **73**, 269-278 (1957).

36. Mandels, M., Andreotti, R., and Roche, C. *Biotechnol. Bioeng. Symp.* **6**, 21-33 (1976).

37. Margaritis, A. and Merchant, R.F.J. *CRC Crit. Rev. Biotechnol.* **4**, 327-367 (1986).

38. Mohagheghi, A., Grohmann, K., Himmel, M.E., Leighton, L., and Updegraff, D.M. *Int. J. of Systematic Bacteriol.* **36**, 435-443 (1986).

39. Mohagheghi, A., Grohmann, K., and Wyman, C.E. *Biotechnol. Bioeng.* **35**, 211-216 (1990).

40. Moldoveanu, N. and Kluepfel, D. *Appl. Environ. Microbiol.* **46**, 17-21 (1983).

41. Montenecourt, R.M. and Eveleigh, D.E. *Appl. Environ. Microbiol.* **34**, 81-85 (1977).

42. Meyer, H.P. and Humphrey, A.E. *Chem. Eng. Commun.* **19**, 149-154 (1982).

43. Park, W.S. and Ryu, D.D.Y. *J. Ferment. Technol.* **61**, 563-571 (1983).

44. Philippidis, G.P. SERI Milestone Report "Complete Initial Phase of SSF Model" (1990).

45. Reese, E.T., Mandels, M., and Weiss, A.H. in *Advances in Biochemical Engineering*, Vol. 2 (eds. Ghose, T.K., Fiechter, A., and Blakebrough, N.), Springer-Verlag, Berlin, 181-200 (1972).

46. Reese, E.T. and Mandels, M. *Biotechnol. Bioeng.* **22**, 323-335 (1980).

47. Robison, P.D. *Biotechnol. Lett.* **6**, 119-122 (1984).

48. Ryu, D., Andreotti, R., Mandels, M., Gallo, B., and Reese, E.T. *Biotechnol. Bioeng.* **21**, 1887-1903 (1979).

49. Ryu, D.D.Y and Mandels, M. *Enzyme Microb. Technol.* **2**, 91-102 (1980).

50. San Martin, R., Blanch, H.W., Wilke, C.R., and Sciamanna, A.F. *Biotechnol. Bioeng.* **28**, 564-569 (1986).

51. Schaffner, D.W. and Toledo, R.T. *Biotechnol. Bioeng.* **37**, 12-16 (1991).

52. Schell, D.J., Hinman, N.D., Wyman, C.E., and Werdene, P.J. *Appl. Biochem. Biotechnol.* **24/25**, 287-297 (1990).

53. Shiang, M., Linden, J.C., Mohagheghi, A., Rivard, C.J., Grohmann, K., and Himmel, M.E. *Appl. Biochem. Biotechnol.* **24/25**, 223-35 (1990a).

54. Shiang, M. and Linden, J.C. Proceedings of the Ethanol from Biomass Annual Review Meeting, SERI (1990).

55. Shinde, S.R. and Gangawane, L.V. *Indian bot. Reptr.* **5**, 57-60 (1986).

56. Shoemaker, S.P., Raymond, J.C., and Bruner, R. in *Trends in the Biology of Fermentations for Fuels and Chemicals* (eds. Hollaender, A., Rabson, R., Rogers, P., San Pietro, A., Valentine, R., and Wolfe, R.), Plenum Press, New York, 89-109 (1981).

57. Tangnu, S.K., Blanch, H.W., and Wilke, C.R. *Biotechnol. Bioeng.* **23**, 1837-1849 (1981).

58. Van Tilbeurgh, H., Pettersson, L.G., Bhikabhai, R., De Boeck, H., and Claeyssens, M. *Eur. J. Biochem.* **148**, 329-334 (1985).

59. Vohra, R.M., Shirkot, C.K., Dhawan, S., and Gupta, K.G. *Biotechnol. Bioeng.* **22**, 1497-1500 (1980).

60. Warzywoda, M., Chevron, F., Ferre, V., and Pourquie, J. *Biotechnol. Bioeng. Symp.* **13**, 577-580 (1983).

61. Watson, T.G., Nelligan, I., and Lessing, L. 1984. *Biotechnol. Lett.* **6**, 667-672 (1984).

62. Wheatley, M.A. and Phillips, C.R. *Biotechnol. Lett.* **5**, 79-84 (1983).

63. Wood, T.M., Wilson, C.A., McCrae, S.I., and Joblin, K.N. *FEMS Microbiol. Lett.* **34**, 37-40 (1986).

64. Yamane, K., Yoshikawa, T., Suzuki, H., Nisizawa, K. *J. Biochem.* **69**, 771-780 (1971).

Table 1. Representative list of data from cellulase production fermentations carried out by cellulase-secreting microorganisms under various operational modes and conditions (N/A denotes unavailable data)[1].

ORGANISM	MODE[2]	CONDITIONS	TIME[3] (days)	MEDIA[4]	SUBSTRATE[5] (carbon source)	PRODUCTIVITY[6] (IU/L/h)	CONCENTRATION[7] (IU/L)	YIELD[8] (IU/g)	REFERENCE
T.reesei MCG-77	Continuous 2-stage; 15 L	1:T=32, pH=4.5 2:T=28, pH=3.5	.027	Defined	Lactose (50-100 g/L)	90	N/A	N/A	48
T.reesei QM 9414	Batch 150 L	T=28, pH=3.2	8	Peptone	Cellulose (60 g/L)	57	11,000	183	20
A.cellu-lolyticus	Batch 1-2.5 L	T=55, pH=5.2	3.3	Yeast Extract	Cellobiose (5 g/L) & Cellulose (15 g/L)	2.5	69	3.5	53
T.reesei QM 9414	Batch (flasks)	T=25, pH=5.0	21	Defined	Cellulose (10 g/L) & Xylose (10 g/L)	N/A	2,000	100	51
T.reesei RUT-C30	Fed-batch 5 L	T=28, pH=4.8	14	Corn steep liquor	Cellulose (230 g/L) & Xylose (100 g/L)	45.4	12,500	38	40
T.reesei L27	Batch 5 L	T=28, pH=4.8	8	Corn steep liquor	Cellulose (50 g/L)	N/A	6,200	124	52
T. pseudo-koningii	Batch (flasks)	T=27, pH=5.0	10	Defined	Cellulose (10 g/L)	N/A	110	11	28
S.flavo-griseus	Batch (flasks)	T=29, pH=6.8	3	Yeast extr. Peptone	Cellulose (10 g/L)	N/A	480	48	41
T.reesei RUT-C30	Batch (flasks)	T=27, pH=5.5	10	Peptone	Aspen Wood (60 g/L) & Cellulose (8 g/L)	N/A	3,900	57	29

ORGANISM	MODE[2]	CONDITIONS	TIME[3] (days)	MEDIA[4]	SUBSTRATE[5] (carbon source)	PRODUCTIVITY[6] (IU/L/h)	CONCENTRATION[7] (IU/L)	YIELD[8] (IU/g)	REFERENCE
P.pino-philum	Batch 16 L	T=35, pH=3.5-5.0 (no pH control)	10	Peptone	Cellulose (60 g/L) or Straw (60 g/L)	N/A	6,000	100	4
P.pino-philum	Batch 16 L	T=35, pH=3.5-6.0 (no pH control)	3	Corn steep liquor	Cellulose (30 g/L) & Straw (30 g/L)	137	10,000	167	5
T.reesei MCG-77	Batch 5 L	T=30, pH=5.0	5	Soya meal	Straw (steamed) (20 g/L)	31	1,900	95	11
T.reesei NG14	Batch 10 L	T=28, pH>3.0	14	N/A	Cellulose (60 g/L)	45	15,000	250	49
T.reesei RUT-C30	Batch 14 L	T=25, pH>5.0	8	Peptone	Cellulose (50 g/L)	N/A	14,400	288	57
T.reesei RUT-C30	Batch 14 L	T=28, pH=5.0	7	Defined	Cellulose (20 g/L)	29	4,200	210	23
"	Continuous 1-stage; 14 L	"	.046	"	"	97	2,100	105	23
"	Continuous 2-stage; 14 L	"	.017	"	"	58	3,300	165	23
"	Fed-batch 14 L	"	8.3	"	Cellulose (100 g/L)	247	22,400	224	23
T.reesei RUT-C30	Fed-batch 20 L	T=25, pH>4.0	11.4	Defined	Pulp cellulose (232 g/L)	427	57,000	246	61

ORGANISM	MODE[2]	CONDITIONS	TIME[3] (days)	MEDIA[4]	SUBSTRATE[5] (carbon source)	PRODUCTIVITY[6] (IU/L/h)	CONCENTRATION[7] (IU/L)	YIELD[8] (IU/g)	REFERENCE
T.reesei RUT-C30	Batch 5 l	T=28, pH=5.0	10	Corn steep liquor	Aspen wood (50 g/L)	50	4,500	90	50
T.thermo-monospora	Continuous 1-stage; 70 L	T=55, pH=7.2	.125	Yeast extract	Cellulose (10g/L) or Cellobiose (5 g/L)	39	310	31	38
T.reesei CL-847	Batch 3,000 L	T=25, pH=5.0	6	Yeast extract	Lactose (60 g/L) & Cellulose (5 g/L)	N/A	10,500	162	60
T.reesei L27	Batch	N/A	8	N/A	Cellulose (80 g/L)	94	18,000	225	56

[1] Cellulase activity is expressed in international units (IU) of activity on filter paper; one IU of cellulase activity on filter paper is the amount of enzyme that yields 1 μmol of equivalent glucose per minute. (Mandels et al., 1976).

[2] Refers to the operational mode of the cultivation (batch, fed-batch, continuous).

[3] For continuous operations, the dilution rate is given (h^{-1}).

[4] "Defined media" refers to media consisting of defined ingredients; otherwise, the rich (undefined) ingredient is given.

[5] For fed-batch operations, the total amount (per unit working volume) of carbon source provided is given. For continuous operations, the concentration of the carbon source in the feed is given.

[6] Cellulase productivity (volumetric production rate).

[7] Cellulase concentration.

[8] Yield of cellulase (IU) per unit mass (g) of substrate consumed. The yield has been calculated by dividing the cellulase concentration by the substrate concentration. It is an approximate value, since in the calculation it has been assumed that all the provided substrate has been completely consumed.

Figure 1. Schematic overview of the biomass-to-ethanol conversion process.

Figure 2. Breakdown of the fixed capital cost of biomass-to-ethanol conversion plant.

Figure 3. Breakdown of the final ethanol cost.

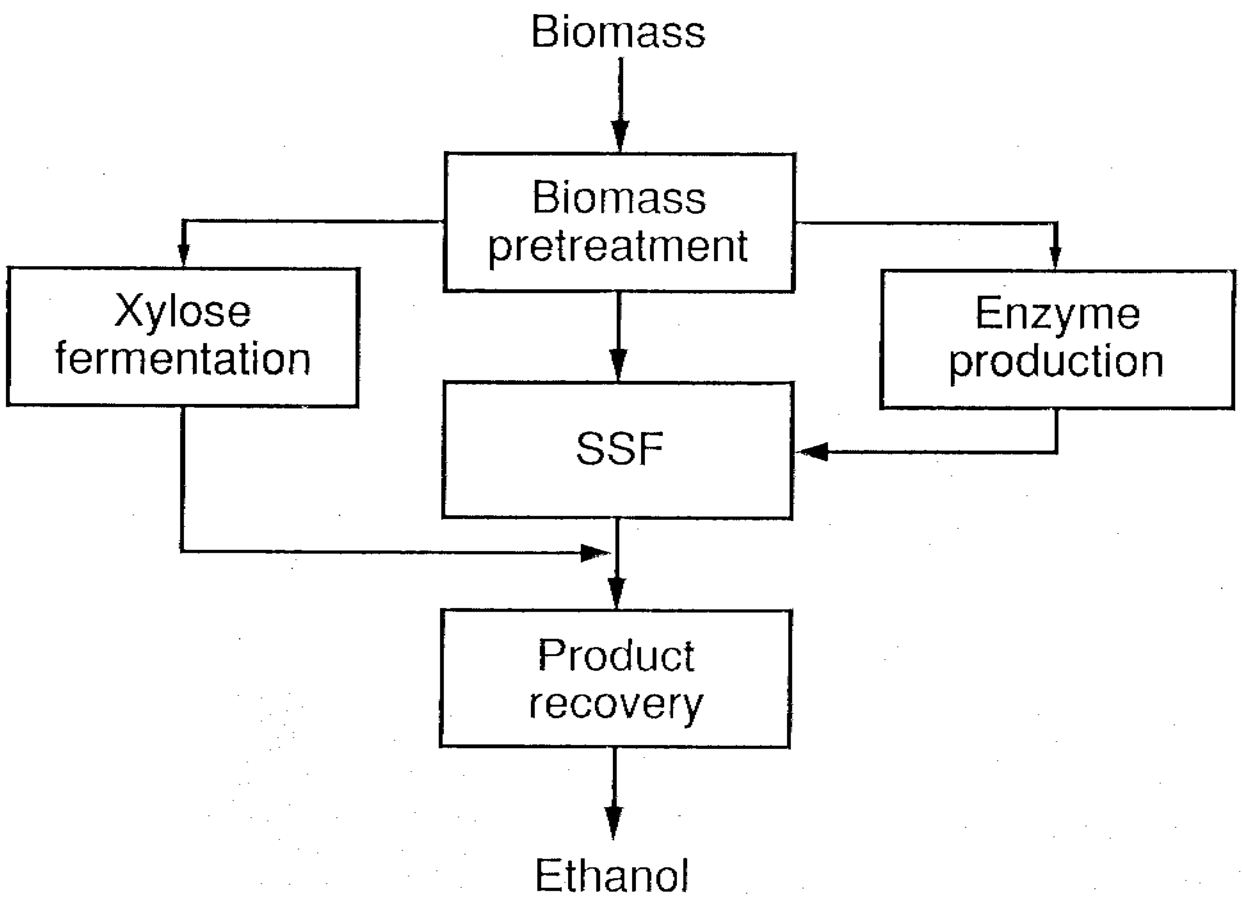

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Fig. 1

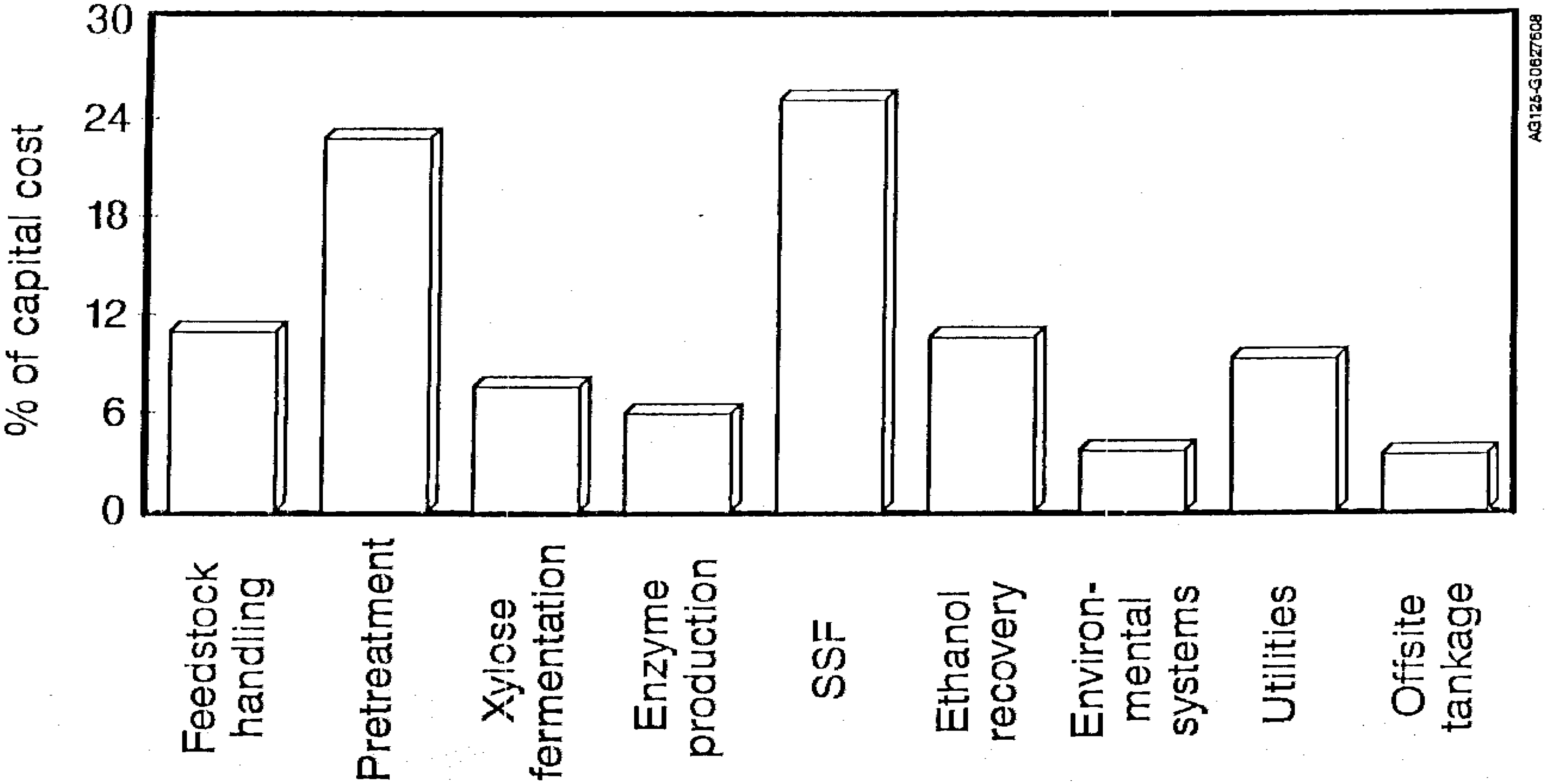
% of capital cost
30
24
18
12
6
0
Feedstock handling
Pretreatment
Xylose fermentation
Enzyme production
SSF
Ethanol recovery
Environ-mental systems
Utilities
Offsite tankage
A9125-G0827608

Fig. 2

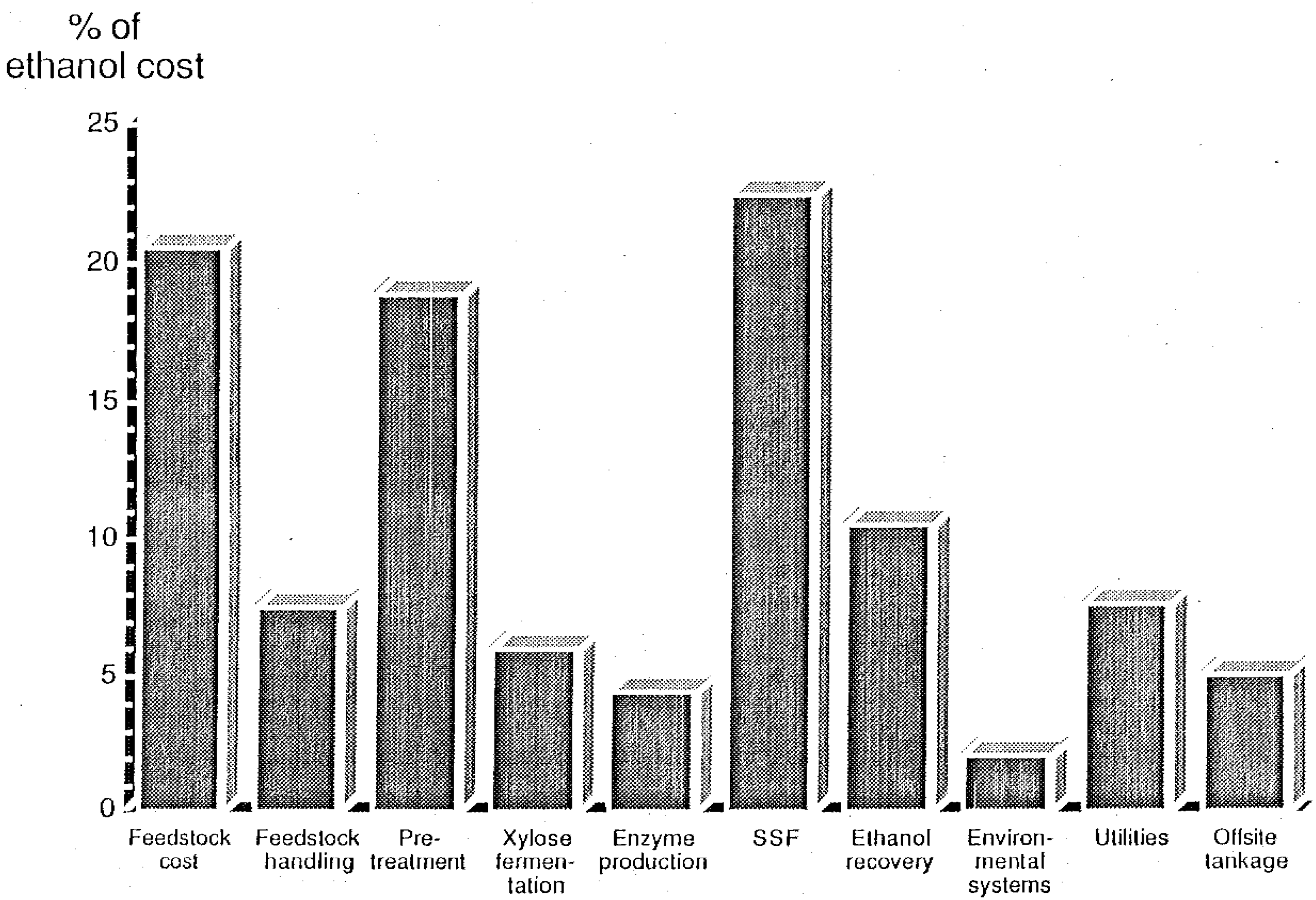

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Fig. 3